

# Clinical Evaluation of a Vaccinia-Vectored Hantaan Virus Vaccine

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We evaluated a vaccinia-vectored vaccine for hemorrhagic fever with renal syndrome in clinical trials. A Phase I dose-escalation study in 16 volunteers divided into four groups demonstrated that subcutaneous inoculation of approximately  $10^7$  plaque-forming units of the recombinant virus was safe and immunogenic. Vaccination of a fifth group of 12 volunteers indicated that neutralizing antibody titers to both vaccinia virus and Hantaan virus were enhanced after a second inoculation. Comparing two routes of vaccination showed that scarification effectively induced neutralizing antibodies in vaccinia virus-naïve volunteers but that subcutaneous inoculation was superior to scarification in vaccinia virus-immune individuals. A Phase II, double-blinded, placebo-controlled clinical trial was conducted among 142 volunteers. Two subcutaneous vaccinations were administered at 4-week intervals. Neutralizing antibodies to Hantaan virus or to vaccinia virus were detected in 72% or 98% of vaccinia virus-naïve volunteers, respectively. In contrast, only 26% of the vaccinia virus-immune volunteers developed neutralizing antibody responses to Hantaan virus. *J. Med. Virol.* 60:77–85, 2000.

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**KEY WORDS:** Hantaan virus; hantavirus; vaccinia-vectored vaccine

## INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is caused by viruses in the *Hantavirus* genus of the family Bunyaviridae. Hantaviruses persistently infect a variety of rodents and are transmitted in infectious aerosols of their urine, feces, and saliva. Prototype Hantaan virus (HTNV) is carried by the striped field mouse, *Apodemus agrarius*, and is responsible for a severe form of HFRS throughout Asia. Seoul virus (SEOV), which is carried by *Rattus norvegicus*, also causes HFRS in Asia and has been found in rats world-

wide. Two other hantaviruses are known to cause HFRS in Europe: Dobrava-Belgrade virus, carried by *Apodemus flavicollis* (yellow-necked mouse) and Puumala virus, carried by *Clethrionomys glareolus* (bank voles) [reviewed in Schmaljohn and Hjelle, 1997]. The widespread distribution of hantaviruses and the large number of HFRS cases reported each year clearly indicate the need for effective vaccines.

We previously reported the development of a recombinant vaccinia virus (VACV) vaccine for HFRS [Schmaljohn et al., 1992]. The vaccine was produced by inserting the HTNV M segment, encoding the envelope glycoproteins (G1 and G2), and the S segment, encoding the nucleocapsid protein (N), into the thymidine kinase gene of the Connaught vaccine strain of VACV. The HTNV M and S segments were under control of the VACV 7.5-K or 11-K promoters, respectively [Schmaljohn et al., 1992]. In preclinical studies, the HFRS vaccine was similar to licensed VACV vaccines in that it produced small, rapidly healing cutaneous lesions when administered by scarification to nonhuman primates [Schmaljohn et al., 1994].

The protective efficacy of the candidate vaccine was assayed in a hamster infectivity model. Although hantavirus-infected hamsters (and other laboratory animals) do not develop disease, viral antigen and RNA can be observed in their lungs approximately 3 weeks after they are infected [Schmaljohn et al., 1992; Chu et al., 1995]. Using this model, we determined that a single intramuscular injection of our recombinant VACV vaccine protected most hamsters from challenge with HTNV [Schmaljohn et al., 1992]. If the hamsters had been previously immunized with a nonrecombinant VACV, protection from HTNV challenge was slightly reduced; however, a second intramuscular in-

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jection of the vaccine overcame preexisting immunity to VACV and resulted in almost complete protection from HTNV challenge [Schmaljohn et al., 1992]. Based on these results, and additional preclinical safety studies, a final container vaccine was produced by Good Manufacturing Practices for use in clinical studies. Here we report findings of Phase I and Phase II trials of the recombinant vaccine.

## MATERIALS AND METHODS

### Viruses, Cells, and Medium

HTNV, strain 76-118, and Sin Nombre virus (SNV), strain CC107, were propagated in Vero E6 cells (Vero C1008: ATCC CRL-1586) in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS), antibiotics, and amphotericin B.

### Vaccine Construction

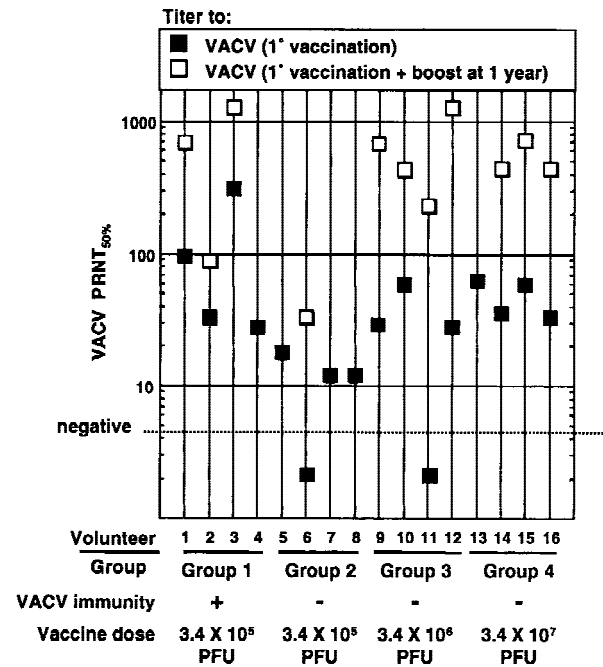
Details of the construction and selection of the recombinant vaccinia-vectored HTNV vaccine were described previously [Schmaljohn et al., 1992]. Briefly, the M segment of HTNV was cloned into the plasmid vector pSC-11 downstream of the VACV 7.5 K promoter [Chakrabarti et al., 1985]. The pSC-11 plasmid contained a LacZ gene under control of VACV 11-K promoter. This gene expresses  $\beta$ -galactosidase and is used for color selection of recombinant viruses. We removed the LacZ gene by digesting with restriction enzymes and replaced it with cDNA corresponding to the S segment of HTNV [Schmaljohn et al., 1992]. Recombinant viruses were generated by homologous recombination between the plasmid and the Connaught vaccine strain of VACV. Initial evaluation of the attenuating properties of the vaccine were performed by inoculation of chorioallantoic membranes of embryonated chicken eggs, and by scarification of nonhuman primates with the vaccine [Schmaljohn et al., 1994].

### Neutralization and Viremia Assays

Sera were diluted serially twofold beginning at a 1:10 dilution in EMEM supplemented with 2% heat-inactivated FBS, antibiotics, and amphotericin B, and mixed with an equal volume of medium containing 50–100 plaque-forming units (PFU) of HTNV per 200  $\mu$ l and 2% normal human serum. The virus-serum mixture was incubated for 1 hr at 37°C and then overnight at 4°C. Selected Phase I samples were assayed by plaque reduction neutralization tests (PRNT) and by a neutralizing enzyme-linked immunosorbent assay (NT-ELISA) simultaneously. Because the results correlated ( $R = 0.82$ ), Phase II samples were assayed only by NT-ELISA.

For HTNV PRNT, confluent monolayers of Vero E6 cells grown in T-25 cm<sup>2</sup> flasks were inoculated with 0.2 ml of the virus-antibody mixture. After adsorption for 1 hr at 35°C, each monolayer was overlaid with 7 ml of 0.6% agarose (SeaKem) containing HEPES-buffered, Eagle's basal medium with Earle's salts, 5% heat inactivated FBS, 1% dimethylsulfoxide (DMSO), and antibiotics. After incubation for 9 days at 35°C, each mono-

A



B

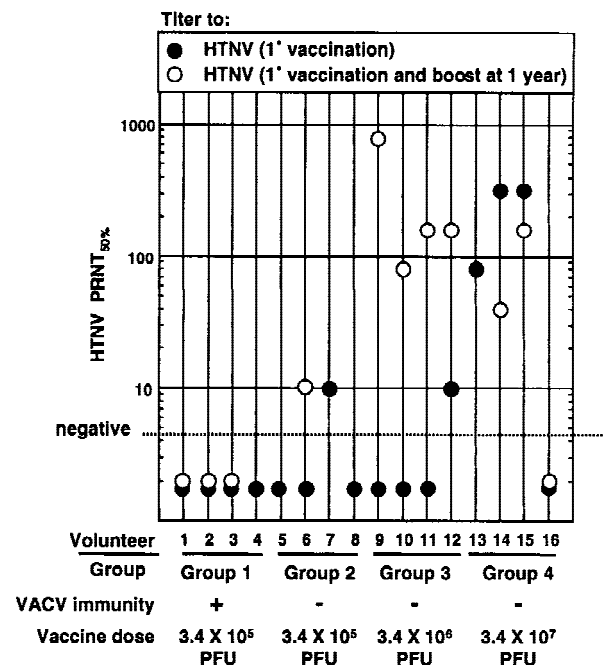


Fig. 1. Neutralizing antibody responses of volunteers in the phase I, dose-escalation study, Groups 1–4, to (A) vaccinia virus (VACV) and to (B) Hantaan virus (HTNV). Volunteers in Group 1 were VACV-immune and volunteers in Groups 2, 3, and 4 were VACV-naïve. Plaque reduction neutralization tests (PRNT) were performed at various times from 7 to 771 days after vaccination. To eliminate accessory factor variation on neutralizing antibody activity, all sera were heated at 56°C for 30 min prior to assay and a constant source of human serum was added to each sample. The highest reciprocal titer measured at any of the assay points is displayed. Volunteers 1, 2, 3, 6, 9, 10, 11, 12, 14, 15, and 16 received a booster vaccination approximately 1 year after the initial vaccination. PRNT titers after the first (1°) vaccination are indicated with closed symbols and those measured after the booster vaccination with open symbols.

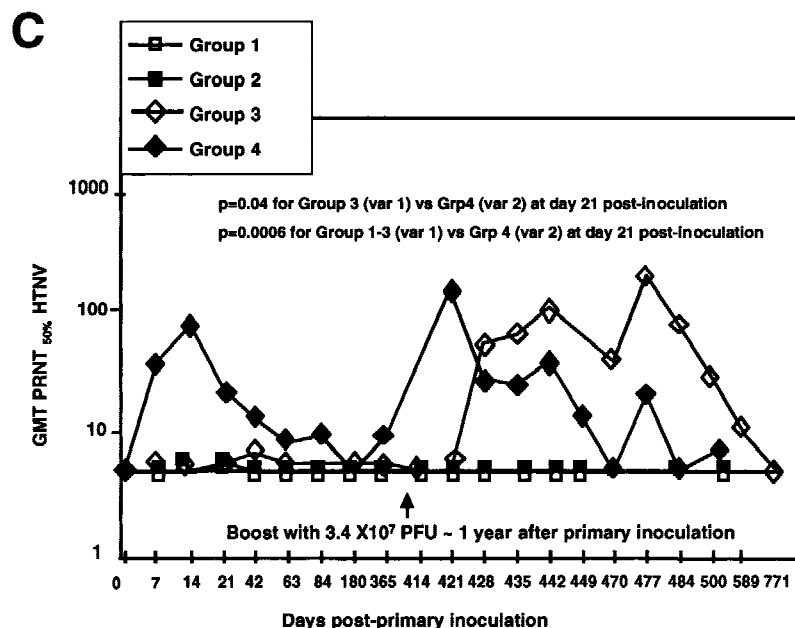


Fig. 1. (C) Geometric mean titers (GMT) of neutralizing antibodies for all individuals in each group at each assay point are shown. The assay lower limit was 1:10 dilution of each serum. For calculation purposes, negative samples were assigned a value of 5.

layer was stained with 3 ml of the original overlay containing 5% neutral red. The flasks were returned to the incubator for one more day and the plaques were counted. All specimens were tested in duplicate. Titers are expressed as the reciprocal of the highest serum dilution that resulted in a 50% reduction in plaque counts (PRNT<sub>50%</sub>). In situ ELISA assays for VACV viremia were performed on blood samples collected periodically from the volunteers as described previously [McClain et al., 1997]. Briefly, aliquots of serum were added to confluent monolayers of Vero cells grown in 24- or 96-well plates. After 1 hr adsorption, fresh medium was added to the wells and the plates were incubated at 37°C for 4–6 days. Cells were then fixed with formalin and incubated with hyperimmune mouse ascitic fluid to VACV followed by enzymatically tagged anti-mouse antibody and a chromogenic substrate (ABTS).

The NT-ELISA for HTNV was performed on monolayers of Vero E6 cells grown in 96-well plates. Wells were inoculated with 0.2 ml of the virus-antibody mixture. Plates were incubated at 35°C in a humidified atmosphere of 5% CO<sub>2</sub> for 9 days. Cultures were decanted and fixed with 0.2 ml of 10% formalin for 15 min at room temperature. Plates were washed and incubated with 0.2 ml of Hanks' balanced salt solution containing 1% normal goat serum for 30 min. The blocking buffer was then removed and 50 µl of a 1:1,000 dilution of HTNV-specific mouse hyperimmune ascitic fluid (HMAF) were added to the appropriate wells for 1.5 hr at 35°C. After the plates were washed three times, 45 µl of a 1:2,000 dilution of peroxidase-labeled anti-mouse IgG antibody were added to all wells and they were incubated for 1.5 hr at 35°C. Plates were then washed five times and 80 µl of ABTS substrate

(Kirkegaard and Perry) were added. After a 30 min incubation at 35°C, OD were read spectrophotometrically at 414 nm. All specimens were tested in duplicate. All NT-ELISA titers are expressed as the reciprocal of the highest serum dilution, which resulted in a 50% reduction in absorbance of the negative controls.

### IgG ELISA

IgG antibodies to HTNV were measured by coating Immulon II plates (Dynatech) with 55 µl per well of sucrose-purified HTNV diluted in phosphate-buffered saline (PBS) (usually 1:50) overnight at 35°C. Plates were blocked with PBS containing 1% normal goat serum. Test sera, diluted 1:100 in blocking buffer, were added to triplicate wells. The plates were incubated for 1.5 hr at 35°C, and were then washed three times. Horseradish peroxidase-labeled anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD) (40 µl/well) was added. After another incubation for 1.5 hr at 35°C, the plates were washed four times and 80 µl of ABTS substrate was added to each well. After 30 min, OD were read spectrophotometrically at 414 nm. OD readings of wells containing human prevaccination sera that were negative for HTNV antibodies were subtracted from test results. OD readings ≥ 0.2 were considered positive.

### Lymphocyte Proliferation Assays

Assays were performed essentially as described previously [Schmaljohn et al., 1995]. Briefly, mononuclear cells were plated at  $2 \times 10^5$  per well in U-bottom plates in the presence of varying dilutions of antigens or controls. Culture medium consisted of serum-free medium (X-VIVO-15) supplemented with 1% fresh autologous plasma. For VACV and mock-VACV antigens, Vero cell

lysates were pelleted through 36% sucrose and infectivity inactivated by irradiating. Antigen dilutions were typically  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  (final concentrations in 0.2-ml cultures). Sucrose gradient-purified HTNV and SNV antigens were tested at dilutions of  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$ . Results shown are from a single "optimal" antigen dilution. The stimulation index (SI) was calculated as test cpm/medium control cpm. Samples incorporating more than 3,000 cpm of  $^3\text{H}$ -thymidine and with  $\text{SI} \geq 3$  were considered positive.

## RESULTS AND DISCUSSION

### Phase I: Dose Escalation

The Phase I study for the HFRS vaccine began as an open label inoculation of four individuals with documented VACV vaccination (Group 1), followed by inoculation of three successive groups of VACV-naïve volunteers with dose escalation (Groups 2–4). All individuals received the vaccine subcutaneously (s.c.). There was a 21-day interval between each group to allow for observation of potential adverse reactions.

Safety was evaluated by the clinical response to VACV and clinical laboratory findings. There were no significant adverse reactions. Two volunteers receiving the  $3.4 \times 10^6$ -PFU dose developed small, cutaneous pox lesions (volunteers 10 and 12, Fig. 1), which healed uneventfully. One recipient of the highest vaccine dose had residual injection site tenderness for 6 days postinoculation. In situ ELISA assays [McClain et al., 1997] were performed to determine if the volunteers became viremic. No VACV was detected in blood samples collected from the volunteers at any time point.

Neutralizing antibodies to HTNV G1 or G2 are known to passively protect rodents from challenge [Schmaljohn et al., 1990]; consequently, elicitation of neutralizing antibody responses is a widely accepted correlate of immunogenicity. To assess immunogenicity of the recombinant vaccine in the dose-escalation study, we measured neutralizing antibody responses to HTNV by PRNT. We also measured neutralizing responses to VACV to gauge infectivity of the vaccine. As expected, all Group 1 volunteers (i.e., who had been immunized previously with VACV) had neutralizing antibodies to VACV at day 0 (data not shown) and after receiving the baseline dose of the recombinant vaccine ( $3.4 \times 10^5$  PFU) (Fig. 1A). None of the volunteers in the VACV-naïve groups had neutralizing antibodies to VACV at day 0, but after vaccination, all but two (volunteer 6 in Group 2, and volunteer 11 in Group 3) developed neutralizing antibodies to VACV (Fig. 1A).

None of the volunteers had neutralizing antibodies to HTNV at day 0. None of the VACV-immune volunteers (Group 1) developed neutralizing antibodies to HTNV after receiving the baseline dose of the recombinant vaccine (Fig. 1B). Neutralizing antibodies to HTNV were detected in one volunteer in each of Groups 2 and 3 and in three of four volunteers in Group 4 (Fig. 1B). Therefore, in this study, the full-strength vaccine dose was superior to lower doses both for VACV infectivity and for eliciting neutralizing antibodies to HTNV.

To examine the anamnestic response to the vaccine, available individuals were revaccinated with the highest dose of the vaccine approximately 1 year after their initial vaccination. Participating in the study were volunteers 1, 2, and 3 from Group 1, volunteer 6 from Group 2, all four volunteers from Group 3, and volunteers 14, 15, and 16 from Group 4. Although only one volunteer in Group 3 had detectable neutralizing antibodies to HTNV after the initial vaccination, all four developed good responses after the booster inoculation of the full-strength dose of vaccine (Fig. 1B). These individuals also all developed higher responses to VACV than were observed after the initial vaccination (Fig. 1A). Similarly, increased titers of neutralizing antibodies to VACV were observed in all three of the revaccinated Group 4 volunteers (Fig. 1A). Two of the three boosted individuals in Group 4 developed rises in antibody titers to HTNV as compared with titers measured immediately before the boost (which were  $\leq 10$ ), but their titers were twofold to fourfold lower than observed after the initial vaccination (Fig. 1B). The other boosted volunteer (volunteer 16) remained negative for neutralizing antibodies to HTNV despite a rise in titer to VACV (Fig. 1A, B).

The geometric mean titers (GMT) of neutralizing antibodies to HTNV were calculated for all bleeds in each group. The Group 4 GMT demonstrated that the highest neutralizing antibody response was achieved approximately 2 weeks after vaccination, and that a rapid anamnestic response appeared upon boosting at 1 year (Fig. 1C). For Group 3, boosting at 1 year resulted in a delayed response, more similar to a primary than an anamnestic response to HTNV (Fig. 1C).

Although cell-mediated immune responses (CMI) to hantaviruses may not be the primary means of preventing infection, it is likely that it plays a role in resolving established infections. In addition, because CMI are more cross-reactive among hantaviruses than neutralizing antibody responses [Asada et al., 1989], measuring CMI might offer insights into cross-protective properties of a vaccine. To assess elicitation of CMI by the recombinant vaccine, we performed lymphocyte transformation (LT) assays. In addition to HTNV and VACV antigen, we measured responses to SNV, an etiologic agent of hantavirus pulmonary syndrome. At day 0, all four individuals in the VACV-immune Group (Group 1) had LT responses to VACV, but none had responses to HTNV or SNV (data not shown). One individual in a VACV-naïve group (volunteer 13, Group 4) had a low SI to VACV at day 0, but all others were negative. Interestingly, at day 0, one person in Group 3 (volunteer 12, Table I) had a proliferative response to SNV ( $\text{SI} = 25.4$ ) and a lesser response to HTNV ( $\text{SI} = 7.4$ ). It is possible that this reflected a pre-existing hantavirus exposure.

After vaccination, one person in Group 3 (volunteer 9) and one person in Group 4 (volunteer 16) failed to develop an LT response to any of the three viruses, but all of the others had positive LT responses to VACV (Table I). Seven volunteers displayed proliferative re-



TABLE I. Lymphocyte Proliferative Responses of Volunteers in Groups 1–4 of the Phase I Dose-Escalation Study of the Recombinant VACV Vaccine for Hantaan Virus

Group VACV immunity Dose	Volunteer	SI			SI, 1-year boost		
		VACV	HTNV	SNV	VACV	HTNV	SNV
Group 1	1	25.8	<3	<3	46.6	3	<3
VACV-immune	2	74.2	3.7	<3	186.4	7.6	7.7
3.4 × 10 <sup>5</sup> PFU	3	49.9	<3	<3	100.9	<3	<3
	4	37	<3	<3	NA	NA	NA
Group 2	5	12.4	<3	<3	NA	NA	NA
VACV-naive	6	66.4	16.6	12.3	34	7.7	5.1
3.4 × 10 <sup>5</sup> PFU	7	4.8	<3	<3	NA	NA	NA
	8	7	<3	<3	NA	NA	NA
Group 3	9	<3	<3	<3	<3	<3	<3
VACV-naive	10	78.6	23.7	<3	53.7	6.7	3.9
3.4 × 10 <sup>6</sup> PFU	11	9.6	12	9.1	10.2	9.1	9.4
	12	128.7	56.7	16.3	18.3	15.9	6.1
Group 4	13	182.8	16.5	8.5	NA	NA	NA
VACV-naive	14	24.1	16.6	6.3	33.5	16	6.4
3.4 × 10 <sup>7</sup> PFU	15	17.3	<3	3.3	4.1	<3	3.2
	16	<3	<3	<3	12.9	8.1	5.1

VACV, vaccinia virus; SI, stimulation index; HTNV, Hantaan virus; Sin Nombre virus; PFU, plaque-forming units; NA, no sample available.

Blood samples were collected from volunteers periodically between days 0 and 84 after the initial vaccination with the recombinant VACV vaccine. Proliferative responses were measured for all volunteers at day 0, and from at least 2 other samplings. Responses to HTNV, SNV, and VACV were measured for each volunteer for at least three different samples. The highest SI achieved at day 42, 56, or 70 after initial vaccination is displayed. For volunteers who received a boost 1 year after their initial vaccination, SI of samples collected between 4 and 6 weeks after the boost are shown.

sponses to HTNV and most of those had responses to SNV (Table I).

The volunteer in Group 4 who initially had no LT response to any of the viruses (volunteer 16) did develop an LT response to all three viruses when boosted 1 year later (Table I). Therefore, although this volunteer did not have neutralizing antibodies to HTNV, a cellular immune response was detected in the lymphocyte proliferation assay.

### Phase I: Comparison of One vs. Two Subcutaneous Inoculations

In our earlier studies, we found that two vaccinations with the recombinant VACV vaccine were better than one for eliciting neutralizing and protective immunity to HTNV in hamsters [Schmaljohn et al., 1992]. To compare the immunogenicity of this vaccine after one or two s.c. inoculations of humans, a group of 12 VACV-naive individuals (Group 5) was randomly divided into two subgroups (5A and 5B). Both groups received a full-strength dose of vaccine ( $3.4 \times 10^7$  PFU), and those in subgroup 5B received a boost of the same dose 42 days later. None of the volunteers had neutralizing antibodies to HTNV or VACV at day 0. After one injection of the recombinant vaccine, 9 of the 12 volunteers developed neutralizing antibodies to VACV (Fig. 2A). Four of the 12 volunteers (volunteers 5 and 6 in group 5A and volunteers 7 and 10 in Group 5B) developed neutralizing antibodies to HTNV (Fig. 2B). After a second inoculation, all 6 volunteers in Group 5B had neutralizing antibodies to both HTNV and VACV (Fig. 2A,

B). As in the first portion of the dose-escalation study, the GMT of neutralizing antibodies to HTNV or VACV peaked at 14 days after the initial vaccination and then declined to baseline levels by day 42 (data not shown). The GMT of the group receiving a booster vaccination peaked at day 7 after boosting and remained at that level for at least four weeks, after which the titer gradually declined (data not shown). Therefore, results obtained for Groups 1–5 indicated that two inoculations were better than one for eliciting neutralizing antibody responses to HTNV or to VACV and that a second inoculation evoked an anamnestic response.

Lymphocyte transformation assays were performed on blood samples collected from individuals in Groups 5A and 5B at days 0, 42, 56, and 63 after vaccination. At day 0, one person showed a weak LT response to HTNV and SNV (volunteer 8, Group 5B, SI < 5), but the rest were negative for the hantaviruses (data not shown). This same individual had a weak LT response to VACV (SI < 5) at day 0 as did volunteers 5 and 6 in Group 5A and volunteer 10 in Group 5B. Volunteer 12 in Group 5B had a moderate LT response to VACV at day 0 (SI = 11). None of these individuals had neutralizing antibody responses at day 0, so we still considered them to be VACV-naive.

After one vaccination all volunteers had LT responses to VACV and all but one in Group 5A (volunteer 2) had LT responses to HTNV and SNV (Table II). The responses to HTNV could be detected in one of these five volunteers at day 42, but not in the other four volunteers until day 63 after vaccination. Similarly,

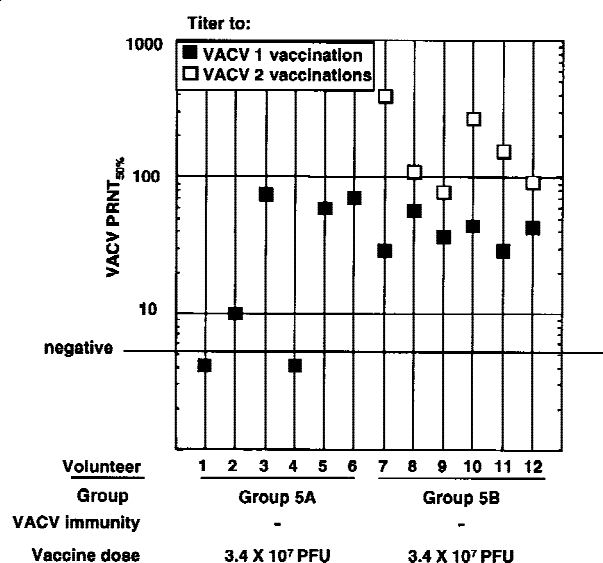
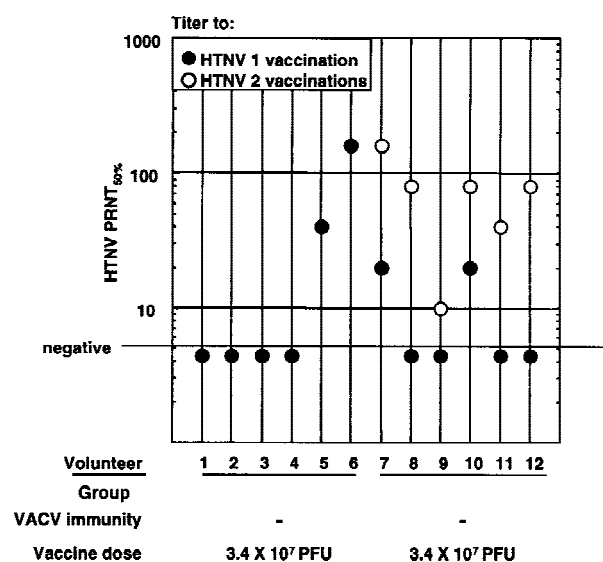
**A****B**

Fig. 2. Comparison of neutralizing antibody titers of volunteers receiving one subcutaneous (s.c.) inoculation (Group 5A) and two s.c. inoculations (Group 5B) of the recombinant vaccine. Periodic blood samples were collected from volunteers from day 0 to day 365 after vaccination. The highest reciprocal neutralizing antibody titer measured at any of the assay points to vaccinia virus (VACV) (A) or Hantaan virus (HTNV) (B) are displayed.

two individuals in group 5B had LT responses at day 42 (before they received a booster vaccination) and all 6 had LT responses at day 63 (after they received a booster inoculation) (Table II).

### Phase I: Comparison of Subcutaneous Inoculation and Scarification

Subcutaneous delivery of the vaccine was intended to avoid the development of cutaneous pox lesions and inadvertent spread of the virus. Nevertheless, four individuals in Groups 1–5 did develop cutaneous lesions.

TABLE II. Lymphocyte Proliferative Responses of Volunteers in the Phase I Comparison of One vs. Two Subcutaneous Inoculations of the Recombinant VACV Vaccine for Hantaan Virus

Group	VACV immunity	Dose	Number of vaccinations	Volunteer	SI		
					VACV	HTNV	SNV
Group 5A				1	69.3	7.5	8.4
				2	39.3	<3	<3
VACV-naive				3	109.6	93.3	80.2
$3.4 \times 10^7$ PFU				4	27	19.3	10.9
1 vaccination				5	31.8	9.5	5
				6	18.6	15.5	10.7
Group 5B				7	52.4	6.2	5.1
				8	58.1	51.4	54.8
VACV-naive				9	43	5.8	9
$3.4 \times 10^7$ PFU				10	39.9	15.1	12.8
				11	35.2	10.2	7.1
2 vaccinations				12	41.2	10.7	11.5

VACV, vaccinia virus; SI, stimulation index; HTNV, Hantaan virus; SNV, Sin Nombre virus; PFU, plaque-forming units. Lymphocyte proliferation responses were measured on samples collected from volunteers on day 0, 42, 56, and 63 after vaccination with the recombinant VACV vaccine. Individuals in group 5B received a second vaccination at day 42. The highest SI achieved at any of the days to VACV, HTNV, or SNV after vaccination are listed.

This prompted us to design an additional Phase I protocol to compare s.c. inoculation to scarification. The trial consisted of an open-label inoculation of six VACV-naive individuals (Group 1) with the recombinant vaccine by scarification (pricks with a bifurcated needle dipped in vaccine) over the deltoid region of the arm. Twelve additional volunteers with documented histories or evidence of previous VACV vaccination (VACV-immune) were randomized between Groups 2 and 3. Group 2 received the vaccine by scarification and Group 3 by s.c. inoculation. All volunteers received a second inoculation 6 weeks later. The first scarification was administered with three pricks for VACV-naive volunteers, and 15 pricks for VACV-immune volunteers. All volunteers received 15 pricks for the boost. Five of the six volunteers (VACV-naive) in Group 1 developed cutaneous pox lesions typical of VACV vaccines after primary scarification and had neutralizing antibody responses to VACV (Fig. 3A). Four of these individuals developed neutralizing antibody responses to HTNV after one vaccination and all six had neutralizing antibodies after a second scarification (Fig. 3B). In contrast, none of the VACV-immune volunteers who received the vaccine by scarification (Group 2) developed neutralizing antibodies to HTNV (Fig. 3B). Two of the VACV-immune individuals who received the vaccine by s.c. injection (Group 3) developed neutralizing antibodies to HTNV after one injection and three had neutralizing antibodies after the second vaccination (Fig. 3B). For these six individuals, there was not an obvious correlation between developing an immune response to HTNV and having low preexisting antibody titers to VACV. That is, the three volunteers who developed neutralizing antibodies to HTNV after two s.c. inoculations had titers to VACV of 5, 66, and 104 at day

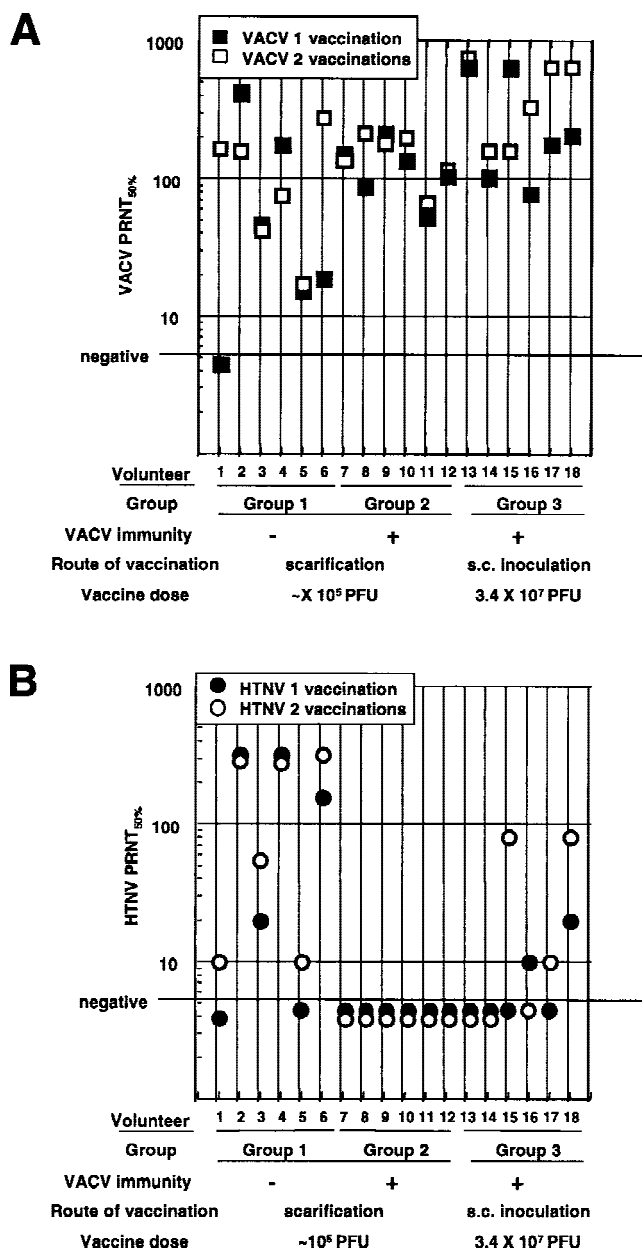


Fig. 3. Comparison of neutralizing antibody responses to Hantaan virus (HTNV) (A) and vaccinia virus (VACV) (B) after vaccination once or twice by scarification or by subcutaneous (s.c.) inoculation. The dose delivered by scarification was approximated based on the amount of liquid that typically adhered to the bifurcated needle. Periodic blood samples were collected from day 0 to day 365 after vaccination and assayed for neutralizing antibodies by plaque reduction neutralization tests (PRNT). The highest reciprocal titer measured for each individual at any of the sampling times are shown.

0 of the study (volunteers 15, 17, 18, respectively, Fig. 3A, B). The volunteers who did not respond to HTNV after two vaccinations had day 0 titers to VACV of 30, 20, and 23 (volunteers 13, 14, 16, respectively, Fig. 3A, B).

Comparing GMT of neutralizing antibodies for the three groups in this study indicated that scarification of VACV-naïve volunteers resulted in the highest neutralizing antibody levels to HTNV with the lowest neu-

TABLE III. Lymphocyte Proliferative Responses of Volunteers in the Phase I Comparison of Scarification and Subcutaneous Inoculation of the Recombinant Vaccine for Hantaan Virus

Group	VACV immunity	Volunteer	SI		
			VACV	HTNV	SNV
Group 1	VACV-naïve	1	118	38.7	5.5
		2	8.1	3.9	<3
		3	18.8	14.5	5.1
		4	47.2	29.7	23.2
		5	<3	<3	<3
		6	8.3	10.3	<3
Group 2	VACV-immune	7	41.6	5.8	6.0
		8	52.6	<3	<3
		9	22.9	<3	3.6
		10	25.5	<3	4.9
		11	21.7	<3	<3
		12	17.4	3.5	3.0
Group 3	VACV-immune	13	13.5	4.9	5.6
		14	42.5	6.3	<3
		15	45.2	<3	10.1
		16	26.2	7.0	6.5
		17	92.2	8.6	4.1
		18	14.8	6.4	3.3

VACV, vaccinia virus; SI, stimulation index; HTNV, Hantaan virus; SNV, Sin Nombre virus.

Lymphocyte proliferative responses were determined for samples collected on days 0 and on at least two of three samples collected at days 55, 93 and 118 after vaccination. The highest SI achieved at any of the time points is shown.

tralizing responses to VACV (not shown). As with s.c. inoculation of VACV-naïve volunteers, scarification of naïve volunteers resulted in peak GMT of neutralizing antibodies to HTNV or VACV 2–3 weeks after vaccination; however, a scarification boost at day 42 did not result in a noticeable anamnestic response to either HTNV or VACV (not shown).

All but one of the VACV-naïve volunteers who were scarified displayed LT responses to VACV and HTNV (volunteer 5) (Table III). However, only two of the scarified VACV-immune volunteers had an LT response to HTNV (Table III). Five of the six VACV-immune volunteers who received the s.c. inoculation developed LT responses to HTNV, although responses were generally lower than those observed in the scarified, VACV-naïve individuals. These data suggest that scarification would be a useful means of inoculating individuals who were not previously immunized with VACV, but s.c. vaccination is better for VACV-immune individuals.

### Phase II Clinical Trial

The Phase II study was double-blinded and placebo-controlled. Of the 142 volunteers enrolled, 74 were VACV-immune and 68 were VACV-naïve (as determined by volunteer's history, absence of vaccination scar, and absence of preexisting antibody to VACV in ELISA and neutralization assays). A total of 115 volunteers received the vaccine ( $3.4 \times 10^7$  PFU) and 27 received a saline placebo. Although all were scheduled to receive two s.c. vaccinations 6 weeks apart, 22 of the vaccinees and four of the controls did not return for a second inoculation. Three volunteers were excluded because of improper vaccine administration; therefore, a

TABLE IV. Neutralizing Antibody Responses of Volunteers in the Phase II Clinical Study of the Recombinant VACV Vaccine for Hantaan Virus

VACV-immunity	Boost	Number of volunteers	HTNV PRNT <sub>50%</sub>			VACV PRNT <sub>50%</sub>		
			% Positive	Range	GMT	% Positive	Range	GMT
Naive	yes	43	72	10–640	160	98	10–640	49
Naive	no	9	44	20–80	40	67	10–68	21
Immune	yes	47	26	10–80	24	100	20–640	270
Immune	no	13	8	10	10	100	16–640	151

VACV, vaccinia virus; HTNV, Hantaan virus; GMT, geometric mean titers; PRNT, plaque reduction neutralization test.

GMTs were calculated by using the highest antibody dilution of each volunteer that reduced the number of HTNV or VACV plaques by 50% (PRNT<sub>50%</sub>) as compared with virus controls not incubated with sera.

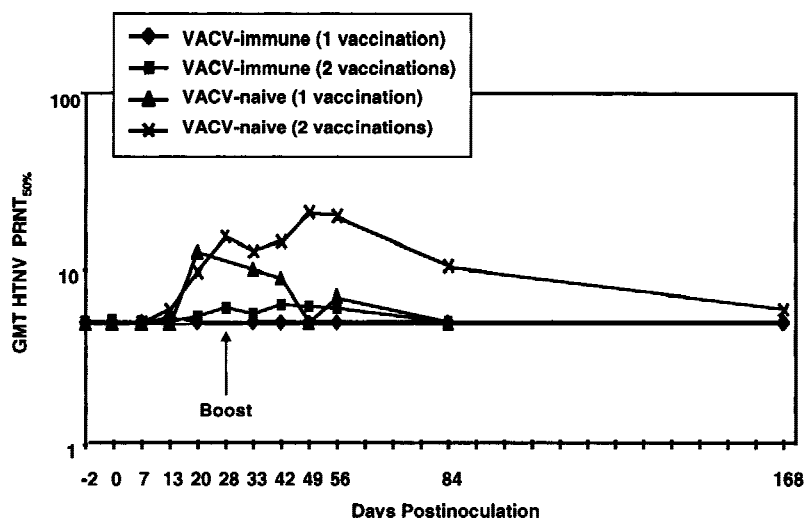


Fig. 4. Geometric mean (GMT) neutralizing antibody responses to Hantaan virus (HTNV) of volunteers enrolled in the Phase II clinical study. Of the 112 volunteers in the final reporting groups, approximately half were already immune to vaccinia virus (VACV-immune) and half were not immune to VACV (VACV-naive). Although all were scheduled to receive two subcutaneous (s.c.) vaccinations 6 weeks apart, 22 subjects did not return for a second inoculation. GMT of neutralizing antibodies to Hantaan virus (HTNV) are shown separately for VACV-immune or VACV-naive volunteers receiving one or two vaccinations.

final group of 112 volunteers who received the recombinant vaccine were assessed (Table IV). Approximately three fourths of the VACV-naive volunteers who received both vaccinations developed neutralizing antibody titers to HTNV; however, only about one fourth of the VACV-immune individuals were PRNT-positive for HTNV after two vaccinations (Table IV). One of 12 volunteers (8%) with neutralizing antibody titers of  $> 40$  to VACV at day 0 developed a neutralizing antibody response to HTNV after two vaccinations (titer = 10). Six of 16 (38%) of volunteers with titers to VACV at day 0 of  $\geq 20 \leq 40$  developed neutralizing responses to HTNV after two vaccinations (range 10–80). Four of 19 volunteers (21%) with day 0 neutralizing antibody titers to VACV of  $< 20$  developed neutralizing responses to HTNV after two vaccinations (range 10–640). Thus there appeared to be a trend toward better responses to HTNV in volunteers with lower pre-existing antibody titers to VACV.

Comparing the GMT of neutralizing antibodies at each time point tested revealed that the peak primary response to HTNV occurred in VACV-naive individuals at 2–3 weeks after vaccination, but declined to baseline levels by 7 weeks if a booster inoculation was not administered (Fig. 4). Likewise, VACV neutralizing antibody levels were higher after the booster inoculation

than after a single inoculation of the recombinant vaccine (data not shown).

As in the Phase I studies, a minority of vaccinees developed cutaneous pox lesions. In the VACV-immune group, none of three volunteers who developed pox lesions had neutralizing antibodies to HTNV. In the VACV-naive group, 9 of 11 (78%) of the volunteers who developed lesions had neutralizing antibodies to HTNV. The GMT of neutralizing antibodies to HTNV for these 9 vaccinees was 80. For individuals who did not develop pox lesions, 68% of VACV-naive individuals had neutralizing antibodies to HTNV, with GMT of 160. Hence, there was no clear indication in the Phase II study that development of a cutaneous pox lesion correlated with a higher neutralizing antibody response to HTNV.

To determine if PRNT reflected seroconversion of all of the volunteers, IgG ELISA was performed with purified, inactivated HTNV as antigen and a 1:100 dilution of each serum. Unlike PRNT, ELISA was expected to detect antibodies to nucleocapsid protein and non-neutralizing antibodies to G1 or G2. In addition to those individuals who displayed neutralizing antibodies, the ELISA detected five VACV-naive and four VACV-immune volunteers with antibodies to HTNV (all of whom had received two vaccinations). Thus an-



tibody responses measured by PRNT or ELISA to HTNV were detected in 84% of VACV-naïve and in 32% of the VACV-immune volunteers who received two vaccinations.

### CONCLUSION

These data indicate that the recombinant vaccine is safe and well tolerated in healthy volunteers. Two vaccinations of VACV-naïve individuals resulted in good seroconversion rates and elicitation of neutralizing antibodies to HTNV. However, in the Phase II study, neutralizing antibodies were not consistently high, and declined to near baseline levels over 3–6 months. Because VACV is no longer routinely administered for prevention of smallpox, it is likely that this vaccine would be efficacious in a target population of VACV-naïve young adults.

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